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Genomic organization and expression of *parkin in Drosophila melanogaster*

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Abbreviations: ARJP, autosomal recessive juvenile parkinsonism; PD, parkinson's disease

Abstract

We report here the isolation, characterization on genomic structure and expression of the *D. melanogaster* homolog of human *parkin.* The 2,122 bp *parkin* gene sequence contains six exons that form a 1,449 bp transcript encoding a protein of 482 amino acids. 151 bp of 5' and 112 bp of 3' untranslated regions were identified by a combination of 5'-RACE/primer extension and 3'-RACE, respectively. The 5' UTR contains three transcription initiation sites. Neither a classical TATA nor a CAAT box was found in the putative promoter sequence. However, binding sites for AhR-Arnt, AP4, NF1 and GATA transcription factors were identified. Transient transfection analysis of the 5' UTR confirmed its promoter activity in HEK 293 cells and SH-SY5Y neuronal cells using a dual luciferase reporting system. The amino acid sequence of *D. melanogaster* Parkin exhibits 42%, 43% and 43% identity to that of human, mouse and rat, respectively, representing a 54 kDa protein band via western blot analysis. It shows a high degree of conservation in the Ubiquitin-like domain at the N-terminus (34%), the In-Between RING finger domains (IBR, 65-69%), and the RING finger domains at the C-terminus (56-57%). The expression pattern of *D. melanogaster parkin* varies during the developmental stages, with the highest expression in the adult stage as measured by competitive RT-PCR. From immunostainings of the embryo, *D. melanogaster parkin* was expressed slightly higher in the central nervous system (brain and nerve cord) during the late embryonic stage.

Keywords: ARJP; *Drosophila melanogaster*; *parkin*; Parkinson's disease; Ubiquitin-ligating enzyme

Introduction

Parkinson's disease (PD, MIM 168600) is a common neurodegenerative disease which has a worldwide distribution and a characteristic movement disorder called parkinsonism in an aged population. The overall prevalence of Parkinson's is 1:1,000, but the incidence increases to 1.4% among individuals 55 years or older and to 3.4% among individuals age 75 or older (De Rijk *et al*., 1997). The disease is caused by striatal deficiency of dopamine which primarily results from neuronal death in the substantia nigra (Olanow and Tatton, 1999).

Most cases of PD occur sporadically as an idiopathic form, but there are also hereditary forms. One of which, autosomal recessive juvenile parkinsonism (ARJP), is a clinically distinct entity characterized by typical PD features but with an earlier ($<$ 40 years) age of onset. Other characteristics include the presence of foot dystonia, a benefit from sleep, a marked response to L-dopa therapy and the absence of Lewy bodies at autopsy (Yamamura *et al*., 1973; Takahashi *et al*., 1994; Forno, 1996; Ishikawa and Tsuji, 1996; Mori *et al*., 1998). Some families with ARJP have allowed genetic mapping and chromosomal localization of candidate regions for the disease (Polymeropoulos *et al*., 1996; 1997; Matsumine *et al*., 1997). Among the genes implicated in familial PD, the largest number of mutations have been found in parkin (gene locus PARK2, MIM 602544), which has been mapped to human chromosome 6q25-27 (Matsumine *et al*., 1997). Parkin has moderate similarities to ubiquitin at the amino terminus as well as a RING finger motif at the carboxyl terminus. Although Lewy bodies have not been identified in the brain of ARJP

patients, antibody staining against Parkin or ubiquitin may still reveal sites of protein aggregation at the microscopic level (Hattori *et al*., 1998b; Kitada *et al*., 1998; Lcking *et al*., 2000).

Human *parkin* consists of 12 exons spanning over 1.5 Mb in length and encodes a 52 kDa protein of 465 amino acids (Hattori *et al*., 1998a; b; Kitada *et al*., 1998; Abass *et al*., 1999). A major transcript of 4.5 Kb was detected in a wide variety of human tissues (Kitada *et al*., 1998). Subsequent studies have demonstrated that *parkin* is expressed in certain neuronal cells of the central nervous system *in vivo* (Horowitz *et al*.,1999; Huynh *et al*., 2001) and functions as a ubiquitin-protein ligase *in vitro* (Imai *et al*., 2000; 2001; Shimura *et al*., 2000), implying that Parkin suppresses neuronal cell degeneration by ubiquitinating misfolded proteins as an E3 enzyme. The expression of *parkin* in bovine peripheral nerve was investigated by RT-PCR and immunoblot analysis. These results point to diverse roles of *parkin* not only in the central but also in the peripheral nervous system (Asako *et al*., 2002).

The mouse cDNA is homologous to human *parkin* which contains a 1,392 bp open reading frame encoding a 464 amino acid (~55 kDa protein). The amino acid sequence of mouse Parkin exhibits 83.2% identity to human Parkin, including the ubiquitin-like domain at the N-terminus (89.5% identity) and the RING finger- like domain at the C-terminus (90.6%). Northern blot analysis revealed that the mouse *parkin* is expressed in various tissues (Kitada *et al*., 2000). Partial cDNA coding for the rat homolog of *parkin* has also been isolated and sequenced. The 1.46 Kb cDNA clone contains a 1,376 bp coding region that also shares strong similarities with human *parkin* cDNA. RT-PCR and *in situ* hybridization showed widespread expression of *parkin* in the rat brain and in the periphery (D' Agata *et al*., 2000).

Recently, to gain insight into the molecular mechanism responsible for selective cell death in ARJP, *Drosophila* model was created, indicating that *Drosophila parkin* null mutants exhibited reduced lifespan, locomotor defects and male sterility with apoptotic muscle degeneration and mitochondrial pathology (Greene *et al*., 2003). And in *Drosophila*, Parkin degraded putative G protein-coupled transmembrane polypeptide (Pael-R, Imai *et al*., 2001) and suppressed its toxicity, which caused age-dependent selective degeneration of *Drosophila* dopaminergic neurons as a Parkin substrate in the molecular pathway of PD (Yang *et al*., 2003).

In the light of the involvement of *parkin* expression levels in ARJP, elucidating the transcription control mechanisms of this gene is of great interest. However, the gene organization and regulatory region have not been well characterized. In this study, the organization and expression of *parkin* is described in the *D. melanogaster* model system.

Materials and Methods

D. melanogaster stocks

Canton-S (Wild type of *D. melanogaster*) strain was kept in a 250 ml glass bottle on a diet of cornmeal, dried yeast, sucrose and agar at 25°C and in 60% relative humidity. Adult flies, pupae, larvae and embryos were harvested and stored at -70°C until use.

Cloning and sequencing of *D. melanogaster parkin* genomic and cDNA

D. melanogaster parkin genomic and cDNA were amplified by PCR and RT-PCR, respectively, using two primers based on the alignment between *Drosophila* genome database and the *parkin* sequences previously reported in human, mouse, rat and bovine [Genbank Accession Numbers: AB009973, AB019558, NM_020093 and AB060701, respectively]: P1, 5'-TATAACAAATGAGTTTTATTTT-3' (forward primer) and P2, 5'-TGTACAACGATGACGAAGGAT-3' (reverse primer) as shown in Figure 1. The PCR reaction was performed as follows, 95°C for 1 min 30 s and then 40 cycles of 95°C for 1 min, 50°C for 1 min 30 s, 72°C for 1 min 30 s and 72°C for 10 min, using *Taq* DNA polymerase (Promega) in a final volume of 50 l. The PCR products were purified using the Wizard PCR Prep purification system (Promega), ligated into the pGEM-T easy vector (Promega), and transformed into *E. coli* competent cells, JM109 (Promega). The *parkin* clones were confirmed by Colony-PCR and sequenced. For automatic sequencing, the BigDye™ Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer) was used according to the manufacturer's instruction on the ABI PRISMTM 377 DNA sequencer (Applied Biosystems). Nucleotide and amino acid sequences of *D. melanogaster parkin* were aligned with that of human, mouse and rat using the Clustal W program and deposited into GenBank. The amino acid sequence of *D. melanogaster parkin* was scanned for functional motifs using ScanProsit (http://www.us.expasy.org/tools/scanprosite).

3' rapid amplification of cDNA ends (RACE)

3' RACE was performed using the 3' RACE system for Rapid Amplification of cDNA Ends (Invitrogen). The first strand of cDNA was synthesized with a (3'-) adaptor primer (included in the kit) and amplified with AUAP (in the kit) and P5 primer as shown in Figure 1. To increase the specificity in the amplification reaction, a secondary nested PCR was performed

with the nested gene specific primer P4 instead of the gene specific primer P5 (Figure 1). Purified products were sequenced as above and the sequence was deposited into GenBank.

5' RACE and primer extension

To characterize the 5' UTR of *D. melanogaster parkin*, 5' RACE was performed according to the manufacturer's instructions of the 5' RACE system for Rapid Amplification of cDNA End (Invitrogen). Briefly, poly(A) RNA was reverse transcribed with primer P6 (Figure 1) and the cDNA was dC-tailed with terminal deoxynucleotidyl transferase and amplified by PCR using primer AAP (in the kit) and P6 as described above. Furthermore, nested PCR was performed to refine these PCR products with primers AUAP and PE (Figure 1). Purified products were directly sequenced and deposited into GenBank. The identification of putative binding sites was performed using the MATINSPECTOR software (Quandt *et al*., 1995). To determine the transcription initiation site of *D. melanogaster parkin*, primer extension analysis was also performed using the Primer Extension System (Promega, Yun *et al*., 2001). The reverse primer PE was labeled with (γ-^{33}P) ATP (3,000 Ci/nmol, Amersham), hybridized with RNA and extended by AMV reverse transcriptase. The DNA-RNA duplex was analyzed on 8% denaturing polyacrylamide gel and autoradiographed.

Luciferase assay

Three constructs, relative to the transcription initiation sites of the *D. melanogaster parkin* gene, were amplified from 5' RACE PCR products containing *parkin* exon 1, using primers (Figure 1) with *Kpn* I and *Hin*d III internal restriction sites for cloning. A series of overlapping promoter fragments were digested with *Kpn* I and *Hin*d III, purified and cloned into the enzyme sites of pGL3-Basic vector (Promega) dephosphorylated (CIAP, Promega). The sequences of these constructs were confirmed by DNA sequencing. The pGL3-Basic vector without insert was also used as a control in the luciferase assay.

FuGene6 (Roche)-mediated transfection of Human embryonic kidney cell (HEK293) and SH- SY5Y neuronal cell were performed as described previously (Jang and Juhnn, 2001; West *et al*., 2001; 2002). Luciferase-containing constructs were cotransfected with pRL-TK vector (Promega) as a control for transfection efficiency, in a molar ratio of 1:100 (pRL-TK vs pGL3). Two days after transfection, cells were rinsed and harvested with Passive Lysis Buffer (Promega). Activities of the firefly luciferase and Renilla luciferase in the cell lysate were measured sequentially using the Dual Luciferase System (Promega) with a luminometer essentially according to the manufacturer's instructions (Luminoskan TL Plus Luminometer, Bio-Rad). Data were expressed as mean±SD of values from three independent experiments.

Competitive RT-PCR

To compare the relative expression level of *D. melanogaster parkin* amongst different developmental stages, competitive RT-PCR was performed using *rp49* as the internal control. Embryo, larva, pupa and adult Canton S were prepared as described above. cDNA was synthesized with oligo (dT) primer (Invitrogen) and amplified by PCR as follows, 95°C for 5 min and then 25 cycles of 95°C for 1 min, 60°C for 1 min 30s, 72°C for 1 min and 72°C for 10 min, using *rp49* primers (GenBank Accession Number U92431, nucleotides 43-62 for forward/436-455 for reverse) as the control and P2 and P3 primer (Figure 1) for *parkin*.

Immunostaining of *D. melanogaster* embryo

Eggs laid by adult flies were collected on media made of agar, grape juice, methyl 4-hydroxybenzoate and dextrose at 25°C. Embryo stage was determined according to the standards of Campos-Ortega and Hartenstein (1985). After the chorion and vitelline membranes of the eggs were removed, fixed, and stained with rabbit anti-Pakin polyclonal antibody (AB5112, Chemicon) according to the manufacturer's instructions for the VECTASTAIN ABC Kit (Vector Laboratories).

Expression plasmid construction and immunoblot analysis

Full length *parkin* cDNA was ligated into prokaryote expression vector pET-30a (+) (Novagen) and the correct insertion was confirmed by Colony- PCR. After transforming the vector into BL21 competent cells (Novagen), IPTG induction was performed for the expression of Parkin. The IPTG-induced Parkin was separated by SDS-PAGE and transferred to PVDF membrane (Millipore) using mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad). The membrane was blocked with 5% (w/v) nonfat dry-milk and incubated with rabbit anti-Parkin polyclonal antibody (AB5112, Chemicon). Parkin was detected using Western Lighting™ Chemiluminescence Reagent Plus (PerkinElmer), with anti-rabbit IgG antibodies conjugated with horseradish peroxidase.

Results and Discussion

Isolation of *parkin* in *D. melanogaster*

Genomic DNA and cDNA clones of *D. melanogaster parkin* were isolated and sequenced. The 2,122 bp

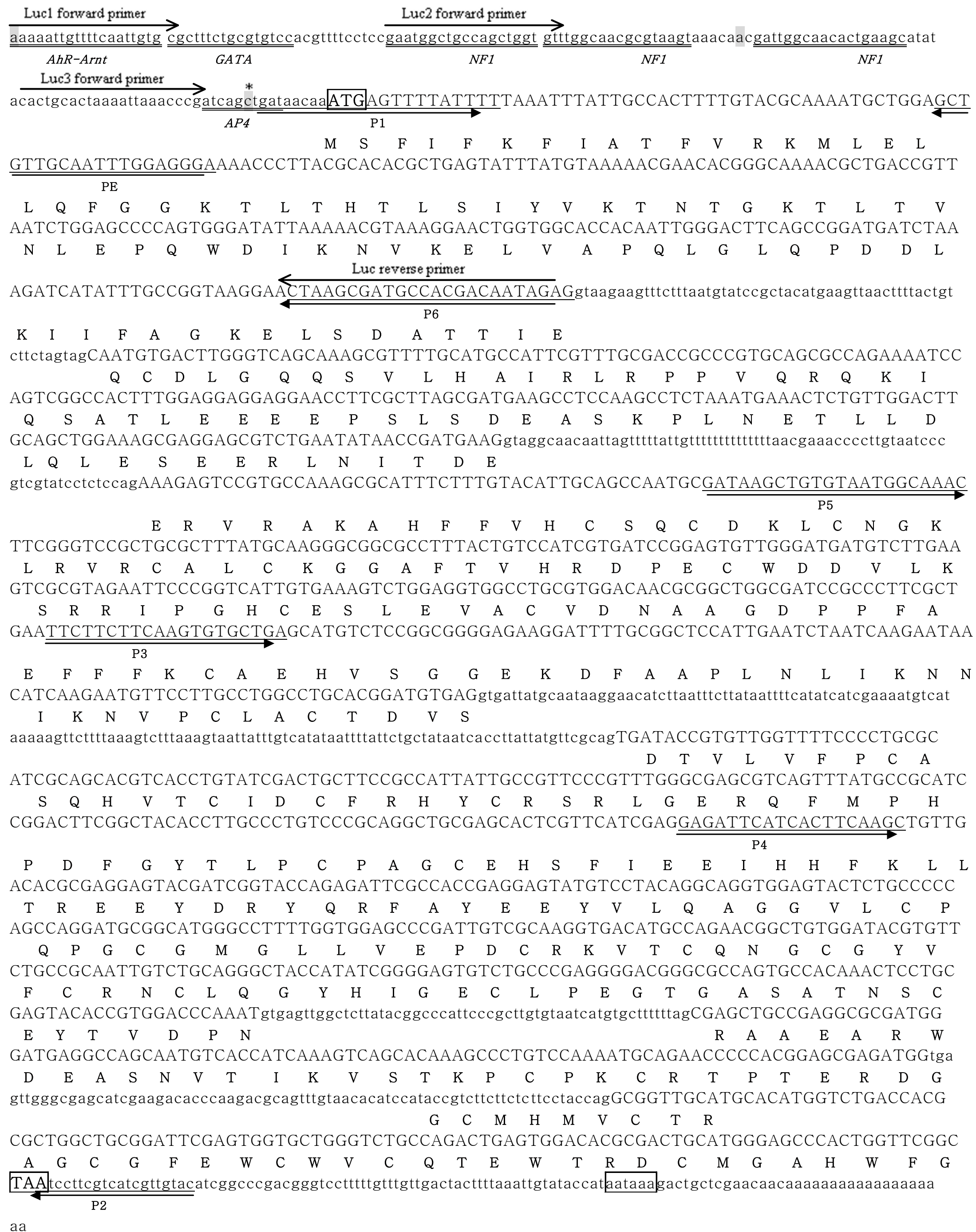

Figure 1. Genomic organization of *D. melanogaster parkin. Parkin* exons are listed in capital letters, while the translation initiation, termination codon and polyadenylation signal are indicated with boxes. Shaded boxes represent transcription initiation sites. The major transcription initiation site, indicated by an asterisk, was designated as the +1 position. The consensus sequences for various transcription factors are shown in italic and underlined. The primers used for the experiments are underlined and denoted with arrows. This sequence was deposited into GenBank (Accession Numbers AF510072, AY093423, AY207374 and AY261675).

Figure 2. Alignment of the deduced amino acid sequence of *D. melanogaster parkin* with human, mouse and rat. The sequences previously reported in human, mouse and rat (GenBank Accession Numbers AB009973, AB019558 and NM_020093, respectively) were compared with that of *D. melanogaster parkin* (GenBank Accession Number AY093423). The conserved domains are in bold and ubiquitin-like domain is underlined. RING domains are shown in double underlining and IBR domain is indicated as a dotted line.

gene sequence of *parkin* is composed of six exons. The 1,478 bp cDNA sequence encodes a protein of 482 amino acids with a calculated molecular weight of 54 kDa. The exon-intron boundaries followed the GT-AG rule for intron splicing, except for the boundary after exons 4 (GT/AT) and 5 (GT/TG). Gene structure analysis indicates that the coding sequence of *D. melanogaster parkin* is encoded by six exons, while the human *parkin* is encoded by twelve exons (Figure 1). The full length of *D. melanogaster parkin* is much shorter in length than that of human because of its strikingly short introns.

The mRNA and gene sequences of *D. melanogaster parkin,* as deposited in GenBank Accession Numbers AY093423 and AF510072 respectively, was aligned with those of human, mouse and rat (Figure 2). The homology in the nucleotide sequence between *D. melanogaster parkin* and human, mouse and rat was 34%, 33% and 34% in each case. When the deduced amino acid sequence was compared, *D. melanogaster* Parkin has a 42% identity with human, and a 43% identity with mouse and rat. As shown in Figure 2, *D. melanogaster* Parkin has a ubiquitin-like domain comprising of 77 amino acid residues (position 30-106) at the N-terminus similar to that of human, mouse and rat. It also contains two C3HC4-

type RING finger domains at the C-terminus and an In-Between RING finger (IBR) domain. RING1, RING2 and IBR domains are located at positions 259-315 (57 residues), 436-476 (41 residues) and 352-394 (43 residues), respectively. IBR, a cysteine-rich domain, has the consensus pattern of C-x(4)-C-x(14-30)-C-x(1-4)-C-x(4)-C-x(2)-C-x(4)-H-x(4)-C. The deduced amino acid sequence of the Ubiquitin-like domain showed a 34% identity when compared with that of human, mouse and rat. The amino acid sequence of the IBR domain has a 69%, 65% and 67% identity when compared to that of human, mouse and rat, respectively. The amino acid sequence of the RING1 domain has a 57%, 56% and 56% identity as compared to that of human, mouse and rat, while the RING2 domain has a 56% identity when compared to that of human, mouse and rat. Such a high degree of conserved homology amongst these different species suggests that these domains are essential for Parkin functions, as previously mentioned by Huynh *et al.* (2001). The RING-IBR-RING domain arrangement was predicted to regulate gene expression (Morett and Bork, 1999), but it is unlikely that Parkin has a function in the nucleus because Parkin is not localized in the nucleus (Kitada *et al.*, 2000). It is more likely that this domain arrangement in *D. melanogaster* Parkin contributes to the interaction with ubiquitin-conjugating enzymes (E2, UbcH7 and Ubch8) as a ubiquitin-ligating enzyme (E3) in the ubiquitin proteasomal pathway. This is the case in human, mouse and rat (D' Agata *et al.*, 2000; Kitada *et al.*, 2000).

Similar to human, mouse and rat Parkin, *D. melanogaster* Parkin possesses several consensus sequences of potential phosphorylation sites for protein kinase C (positions 38-40, 201-203, 428-430, 432-434, 443-445), casein kinase II (83-86, 114-117, 121-124, 133-136, 148-151, 164-167, 237-240, 280-283, 317-320, 329-332, 441-444, 443-446) and cAMP- and cGMP-dependent protein kinase (369-372). But unlike that of human, mouse and rat, there was no phosphorylation sites for tyrosine kinase found in *D. melanogaster* Parkin (Figure 2).

Using the RACE method, 151 bp of 5' UTR (GenBank Accession Number AY261675) and 112 bp of 3' UTR (GenBank Accession Number AY207374) were discovered for the *D. melanogaster parkin* cDNA. These UTR sequences were instrumental in identifying the initiation and termination sites of transcription. The 3' RACE generated a 1,097 bp PCR product, while the nested PCR showed a 638 bp product (Figure 3). The poly(A) site was located 15 bp downstream of a polyadenylation signal sequence. The results indicate that there is no alternative splicing in the 3' UTR and that the 1.7 Kb major transcript was identified as confirmed by Greene *et al.* (2003).

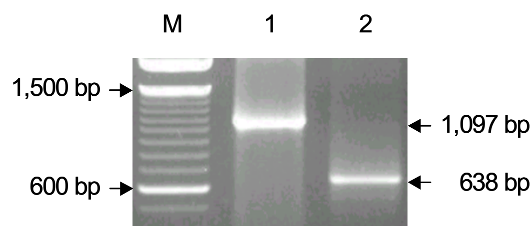

Figure 3. 3' RACE. PCR produced only one product, 1,097 bp in size and nested PCR showed a 638 bp product. The sequence of 3' UTR in *D. melanogaster parkin* was deposited into GenBank (Accession Number AY207374). Lane M, 100 bp DNA ladder (Invitrogen); lane 1, 3' RACE PCR product; lane 2, Nested PCR product.

Identification of transcription initiation sites

The transcription initiation site of *D. melanogaster parkin* was determined using a combination of primer extension and 5' RACE. Primer extension analysis was performed using a PE primer positioned at exon 1. The resulting three bands extended the *D. melanogaster parkin* mRNA. But, an 80 bp size product showed was much stronger, indicating a major transcription initiation site (Figure 4B). We performed 5' RACE analysis to ascertain where transcription initiation sites are present (Figure 4A). 445 bp, 356 bp and 304 bp of primary PCR products were obtained and subsequent nested PCR generated 257 bp, 168 bp and 116 bp products. The estimated transcription initiation sites were mapped at 151 bp, 62 bp and 10 bp upstream of the ATG translation initiation site (Figure 1). The 5'-most initiation site has been designated as the +1 position and corresponds to a cytosine residue at position -10 relative to the ATG initiation codon. Other positions indicated alternative transcription initiation sites and these 5' UTR variants may be generated from the use of alternative promoters. In human *parkin*, two transcription initiation sites were located at 97 bp and 154 bp upstream of ATG using primer extension as described by West *et al.* (2001). Like human *parkin*, consensus TATA and CAAT boxes that could define a transcription initiation site were not found in the putative promoter regions (West *et al.*, 2001). However, the sequences surrounding the three transcription initiation sites contained several sequence elements that were identified by the MATINSPECTOR program (Figure 1). These sequence elements include consensus binding sites for AhR-Arnt, AP4, NF1 and GATA transcription factors that define the transcription initiation site in many TATA-less promoters (Jiang *et al.*, 2000), suggesting that these sites may be functionally involved in the transcriptional control of *D. melanogaster parkin* and in the bi-directional transcription activation within the promoter like human (West *et al.*, 2003). In human, the analysis of *parkin* promoter identified a novel gene of unknown function, *parkin co-regulated gene*

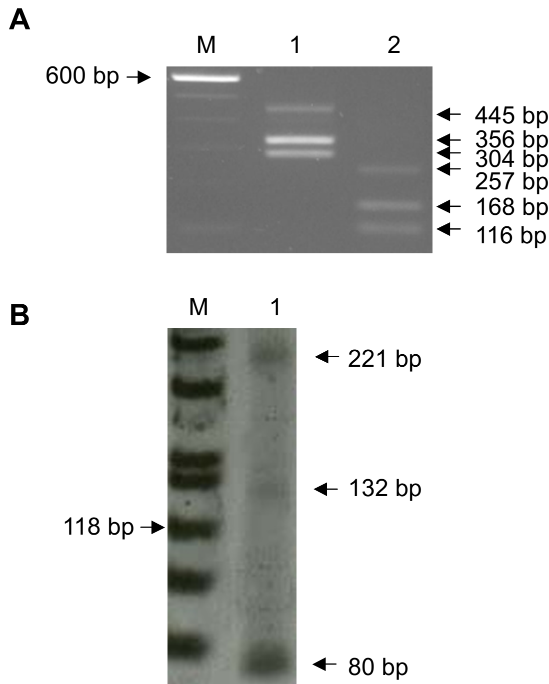

Figure 4. Identification of the transcription initiation sites in *D. melanogaster parkin*. (A) 5' RACE. Amplifications with primers specific for *parkin* produced 445, 356 and 304 bp products (lane 1). A secondary nested PCR showed 257 bp, 168 bp and 116 bp products in size (lane 2). The products were directly sequenced and revealed transcription initiation sites 151 bp, 62 bp and 10 bp upstream of the *parkin* translation initiation codon. Lane M, 100 bp DNA ladder (Invitrogen); lane 1, 5' RACE PCR product; lane 2, Nested PCR product. (B) Primer extension. The assay revealed three extension products corresponding with 5' UTR of approximately 221 bp, 132 bp and 80 bp. Lane M, labeled ΦX174*Hinf* I DNA markers; lane 1, extension products of mRNA, which are indicated by arrows.

(*PACRG*) which shared a common promoter with *parkin* (West *et al*., 2003). And based on the highly conserved *D. melanogaster* homolog of *PACRG*, *D. melanogaster parkin* may interact with *D. melanogaster PACRG* in an uncharacterized biological pathway like human *parkin* (West *et al*., 2003). However, it is unclear which of the transcription factors play a role in the transcriptional regulation of *D. melanogaster parkin* under normal or pathological conditions. Therefore, more work needs to be done on the putative role of these transcription factors in regulating *parkin* expression.

Promoter activity of *parkin* in *D. melanogaster*

To assess whether the 5'-flanking region of *D. melanogaster parkin* could support transcription in mammalian cell lines, three deletion constructs of the *parkin* promoter spanning -141 to +268 bp, -93 to +268 bp and -27 to +268 bp were generated by PCR as described by Jiang *et al*. (2000) and West *et al.* (2001). The same reverse primer (Luc) for all constructs and various forward primers (Luc1, Luc2 and Luc3) were used (Figure 1). These constructs were fused to the luciferase gene in the pGL3-Basic vector. Transient transfection experiments with vectors containing inserts and empty vectors were performed in HEK293 and SH-SY5Y cells. In three replicate experiments, there was significantly higher promoter activity detected in the pGL3-Basic vectors with promoter region inserts as compared to the those without insert (Figure 5), indicating that there is transcription initiations within these promoter regions. Differences were found in the level of expression

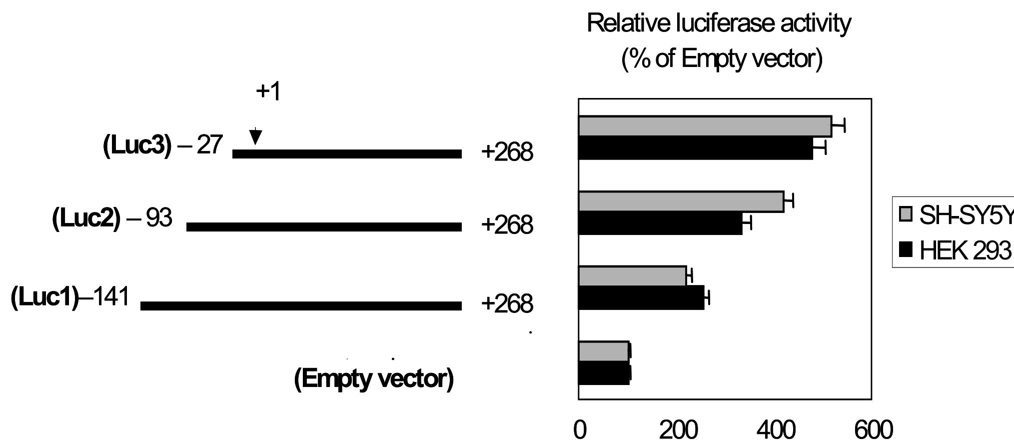

Figure 5. Analysis of the promoter activity of *parkin* by transient expression. On the left is a depiction of the fragments used for the construction in which three *parkin* promoter regions were designed and inserted upstream of the luciferase gene; numbers indicated the position on the promoter sequence of the beginning and the end of each fragment. The major transcription initiation site was designated as +1. Shown on the right are the results obtained by cotransfection of HEK293 (black bar) and SH-SY5Y cells (striped bar) with the corresponding constructs and the control vector. Values of luciferase activity were normalized and represent the mean S.D. of three independent experiments (n = 3, $P < 0.05$).

between the cell lines and amongst the constructs. Although the relative luciferase activity was similar within each cell line, the relative activity was higher in SH-SY5Y cells. Interestingly in both cell lines, the promoter construct spanning -27 to +268 bp revealed a higher promoter activity, while the -27 to -93 bp and -93 to -141 bp constructs showed a significantly decreased activity, indicating that some elements in these regions may down-regulate the expression of *D. melanogaster parkin*. These results suggested that the 5' region of *D. melanogaster parkin* contains regulatory sequences and functions as a promoter of gene transcription. While the regulatory mechanisms controlling *D. melanogaster parkin* transcription are not known, these data represent an important first step in understanding this process.

Expression of *D. melanogaster parkin*

Competitive RT-PCR was performed to quantify the relative expression of *D. melanogaster parkin* amongst the different developmental stages. A 788 bp PCR product was amplified using P2 and P3 primers, while a 413 bp product of the rp49 gene was co-amplified as an internal control (Figure 6). The results showed that parkin was expressed highest in the adult stage followed by the embryonic, larval and pupal stages in decreasing order. To verify the possibility that the expression of *parkin* is differentially regulated during development, we investigated the temporal and spatial expression patterns of *parkin* in *Drosophila* embryos at different developmental stages as shown in Figure 7. Immunostaining of embryos showed that *parkin* was expressed across the whole embryo in early stage 2 (Figure 7A). At stage 5, *parkin* was expressed evenly across the embryo but at a somewhat lower level (Figure 7B). At stage 8, *parkin* was expressed higher at the anterior (cephalic furrow) than other parts of the embryo (Figure 7C). Stage 9 showed higher expression of *parkin* in neuroblast. At stage 10, the procepalon and dorsal regions showed a higher expression of *parkin* particularly at the region around the clypeolabrum. At stage 13, *parkin* showed higher expression in the procephalon and ventral nerve cord regions (Figure 7D). In summary, *parkin* was expressed ubiquitously and at higher levels in the early stages. The expression level was a whole de-

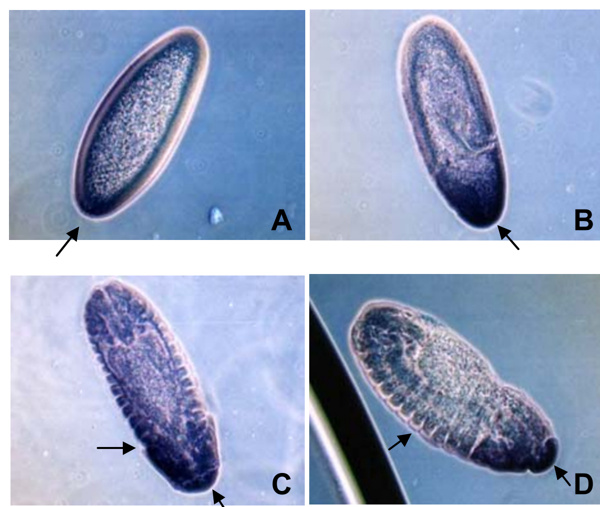

Figure 7. Immunostaining of *parkin* in *D. melanogaster*. Temporal and spatial expression patterns of *parkin* are shown by arrowheads. Embryo was immunostained for Parkin using antibody. (A) embryo at stage 2; (B) at stage 5; (C) at stage 8; (D) at stage 13. Stage of *D. melanogaster* embryo was determined by Campos-Ortega and Hartenstein (1985).

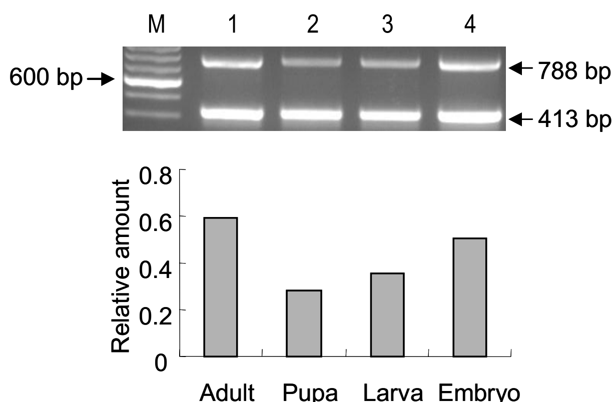

Figure 6. Competitive RT-PCR. Upper and lower bands represent the 788 bp of PCR product for *D. melanogaster parkin* and the 413 bp of product for *rp49* respectively. Lane M, 100 bp DNA ladder (Invitrogen); lane 2, adult; lane 3, pupa; lane 4, larva; lane 5, embryo.

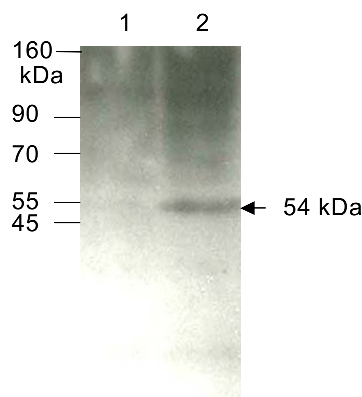

Figure 8. Immunoblot analysis. The IPTG-induced Parkin in *E. coli* was detected with antibody. As a result, one major protein band (54 kDa) which represented the full-length *D. melanogaster* Parkin was identified. Lane 1, without IPTG-induction; lane 2, with IPTG-induction. Molecular weight markers in kDa are shown on the left.

creased in the later stages. But, expression was more concentrated at the central nervous system (brain and nerve cord) in the later stages, which is in line with its function in the central nervous system.

And we performed the immunoblot analysis on IPTG-induced Parkin in *E. coli* using anti-Parkin antibody (Figure 8), which showed one prominent band with a molecular weight of 54 kDa, as described previously (Horowitz *et al*., 2001). In Horowitz *et al*. (2001), Parkin was detected in a diverse set of organisms including *D. melanogaster*, frogs, birds and mouse, indicating that Parkin may play a universal role that has been preserved across different species in evolution. Since *D. melanogaster* is an efficient model for studying genes in human disease, it will be important to study the effects of *parkin* mutations as it relates to PD. *D. melanogaster* gene homolog of human, mouse, rat and bovine genes have yielded invaluable insight into the molecular mechanisms underlying disease and offer exciting possibilities for therapy (Schenk *et al*., 1999).

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